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RAC PROJECT: 195 PL

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DEGRADATION OF ORGANIC CONTAMINANTS BY ANAEROBIC BACTERIA IN LAKE ONTARIO SEDIMENTS

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Final Report on M.O.E. Research Project 195 PL

Degradation of Organic Contaminants by Anaerobic Bacteria in Lake Ontario Sediments

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Abbreviations used in the text: 3-ClBZ, 3-chlorobenzoic acid; 3-BrBZ, 3-bromobenzoic acid; 3-IBZ, 3-iodobenzoic acid; 3,5-diClBZ, 3,5-dichlorobenzoic acid; 4-amino-3,5-diClBZ, 4-amino-3,5-dichlorobenzoic acid; BZA, benzoic acid

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ABSTRACT

The nature of this research study was to investigate the extent of anaerobic biodegradation of halogenated aromatics in sediments collected at pre-selected sites from Lake Ontario along the Toronto Waterfront.

Anaerobic bacteria from sediments collected at different sites in Lake Ontario were cultivated in the presence of halogenated benzoates. Biodegradation was assessed by net gas production (CH_4, CO_2), measured by pressure transducer and gas chromatography (GC), and by substrate disappearance using high pressure liquid chromatography (HPLC).

The ability of anaerobic bacteria in the sediments to adapt to halogenated benzoates and to subsequently cross-adapt to more complex halogenated aromatics was assessed. This was done by suspending the sediments from each site in Revised Anaerobic Mineral Medium (RAMM) in the presence of halogenated benzoates. Total and viable bacterial counts were performed in order to ensure that there was no significant loss during prolonged storage of sediments as necessitated by the experimental plan. To ascertain the types of bacteria present, anaerobic organisms were cultured in roll tubes according to V.P.I. procedures and examined by phase-contrast microscopy.

A characteristic metabolic pattern was observed with preferred biodegdradation of 3-iodobenzoate (3-IBZ) and 3-bromobenzoate (3-BrBZ) over 3-chlorobenzoate (3-ClBZ). Upon addition of further substrates, degradation occurred immediately without a significant lag period. Following adaptation, the sediments were tested for the biodegradation of more complex

halogenated aromatics. 3,5,-Dichlorobenzoate (3,5-diClBZ) was depleted in 3-5 weeks, whereas the amino derivative, 4-amino-3,5-diClBZ, was incompletely degraded even after 16 weeks, following initial adaptation to 3-ClBZ. However, when initially adapted to 3-BrBZ, the 4-amino-3,5-diClBZ was depleted within 6 weeks. The cross- adaptation results thus showed that 3-BrBZ is preferred for initial microbial adaptation. HPLC analyses detected a transient accumulation of benzoic acid in the cultures during biodegradation, indicating a reductive dehalogenation pathway with benzoic acid as the main intermediate.

Bacterial numbers (total counts, viable counts) did not decrease significantly during prolonged storage and were found to be higher than those in nonpolluted sediments of most lakes. Bacteria grown on agar roll tubes and characterized by phase contrast microscopy were highly diverse. The consortium consisted of gram negative and positive cocci, gram negative rods, and spiral forms.

The findings demonstrate a potential for break-down of halogenated contaminants by anaerobic cultures found in Lake Ontario sediment. This points to the feasibility of developing applications for anaerobic degradation of xenobiotics under controlled conditions prior to their release into the environment.

INTRODUCTION

The environmental cycling of carbon compounds within the aquatic environment depends upon a balance being maintained between the synthesis of organic compounds (by plants and animals) and their degradation (by microorganisms). This balance is threatened by the discharge of xenobiotics, potential carcinogens and teratogens. Such pollutants are currently being discharged into freshwater ecosystems with industrial waste and farmland runoff. These contaminants, such as halogenated aromatic pesticides and herbicides, and polychlorinated biphenyls (PCB's) adsorb to particulates, including oil droplets, in lake water, and settle in the sediment. Here they persist and may eventually appear in higher trophic levels.

Recent surveys of Toronto waterfront sediments (Persaud et al., 1985) disclosed that muds in boat slips of the Inner Harbour and in Toronto Island waterways have elevated levels of total volatile residues, solvent extractables, oil, and grease (loss on ignition) and PCB's. Many such compounds were originally believed to be recalcitrant. Within the past few years, however, several research groups (e.g. Tiedje and colleagues, Michigan State University) have found that chlorinated benzoates, breakdown products of PCB's and some herbicide congeners, as well as chlorinated phenols, including wood preservatives, can be decomposed in the laboratory by stringently anaerobic bacterial consortia present in lake sediments (Tiedje et al., 1986). During bacterial transformations of chlorinated aromatics, chlorine atoms are removed from the aromatic ring in a reductive dehalogenation reaction. The dechlorinated products thus formed are less hazardous and more susceptible to further degradation under both aerobic and anaerobic conditions (Suflita et al., 1982, 1983; Horowitz et al., 1983). It is thus possible that some of the highly chlorinated, serious pollutants can be rendered less persistent and less toxic through microbial activity.

In the 1960's it was first recognized that some manmade chemicals released into the environment were recalcitrant and thus disturbing the essential ecosystem balance. Most of the research regarding this issue had concentrated on aerobic biodegradation of pollutants since it was assumed that aerobes have a more versatile metabolism. With the recent improvements in anaerobic methodology, interest was generated in anaerobic metabolism. Anaerobic biodegradation is cost-effective - if the pollutant is bound to soil or sediment, it may be degraded in place by making the site anaerobic (flooding, etc.). Anaerobic waste processing units are often less expensive since the cost for pumping oxygen into the system is avoided and a smaller hydraulic volume is required (Tiedje et al., 1986).

The purpose of this research study was to determine whether halogenated benzoates, simple and more complex, were biodegradable in sediments removed from sites along the Toronto waterfront; and, also to show that the anaerobic consortia responsible for breakdown of simple compounds were able to cross-adapt and degrade polyhalogenated pollutants. The results suggest that cross-adaptation may be an effective approach to a more rapid biodegradation of complex pollutants in lake sediments.

MATERIALS AND METHODS

Equipment purchased

XT compatible computer with two disk drives, 640K, Packard Bell monochromatic monitor and keyboard (Comptronic Computer Centre Ltd., Toronto, Ont.); Brother AX-15 typewriter (Dominion Typewriter Ltd., Toronto, Ont.); Coy anaerobic glove box (Coy Laboratory Products, Ann Arbor, MI); Jenkins mud sampler (Freshwater Biological Association, Dorset, England); Bellco tube spinner (Bellco Glass Inc., Vineland, N.J.),; portable cooler (Canadian Tire Corporation, Toronto, Ont.); Hungate gassing apparatus (Figure 2).

Sediment collection

Sediment samples were collected in Lake Ontario from sites listed in Table 1, (Figure 1A to 1E), by Ponar grab sampler, and transferred to sterile glass jars (0.5 l capacity), filled completely to maintain anaerobic conditions. The jars were tightly capped, and stored at 4°C in the dark.

Preparation of test medium

The Revised Anaerobic Mineral Medium (RAMM) used in this study and the method for its preparation were those developed by Shelton and Tiedje (1984a). The medium contained phosphate buffer (pH 7.0), mineral salts (NH_4Cl, $CaCl_2.2H_2O$, $MgCl_2.H_2O$, $FeCl_2.4H_2O$, $Na_2EDTA.2H_2O$), trace metals ($MnCl_2.2H_2O$, H_3BO_4, $ZnCl_2$, $CuCl_2$, $NaMo_4.2H_2O$, $CoCl_2.6H_2O$, $NiCl_2.6H_2O$, Na_2SeO_3, $Na_2EDTA.2H_2O$), vitamins (biotin, folic acid, lipoic acid, thiamin.HCL, riboflavin, nicotinic acid, pyridoxal.HCL, pantothenic acid, cyanocobalamin, para-aminobenzoic acid),

reducing agent (0.125% L-cysteine.HCL - $Na_2S.9H_2O$, pH 10), and a redox indicator (0.1% resazurin). Hungate technique and apparatus (Figure 2) were used for anaerobic gassing of flasks and for preparation of oxygen-free gases and media. This procedure involved sparging with 10% CO_2 - 90% N_2 gas mixture passed through copper fillings at 300°C to remove traces of oxygen. All methods were essentially those of Hungate (Hungate, 1968).

Preparation of test bottles for total gas production measurement

Slurry was prepared using anaerobic techniques by suspending 500 ml of collected sediment in RAMM in a 1:2 ratio. Wheaton serum bottles, 160 ml capacity, (Wheaton Scientific Products, McGraw Park, Ill.) were filled with 50 ml of RAMM and 50 ml of sediment slurry. After 24 hours the pressure inside each bottle was equilibrated to atmospheric pressure by means of venting with a needle. The following test compounds were added at 0.3 mM, 0.6 mM, 1.2 mM and 2.4 mM: 3-BrBZ, 3-ClBZ, 3-IBZ, benzoic acid (BZA), phenol, cresol, 3,5-diClBZ, 4-amino-3,5-diClBZ and ethanol (positive control). Autoclaved (30 min, 10 lbs./sq. in.) and non-autoclaved control bottles containing 50 ml of slurry and 50 ml of RAMM were also included. The bottles were incubated stationary in the dark. All compounds were tested in duplicate.

Preparation of substrates

Water insoluble test compounds were first dissolved in 1N NaOH. Appropriate amounts of test compounds were dispensed through a disposable membrane filter (Millipore, 0.45 um porosity).

Chemicals

3-BrBZ, 3-ClBZ and cresol were obtained from Sigma Chemical Co., St. Louis, MO.; 3,5-diClBZ and 3-IBZ were supplied by Fluka, Switzerland; 4-amino-3,5-diClBZ was obtained from Pfaltz and Bauer Research Chemical Division, Waterbury, CT.; benzoic acid was obtained from J.T. Baker Chemical Co., St. Louis, MO., phenol from Mallinckrodt Chemical Works, St. Louis, MO., and ethanol from Consolidated Alcohols Ltd., Toronto, Ont.

Measurement of gas production

Total gas by pressure transducer

Total gas production (CH_4 and CO_2) was measured by a pressure transducer (multi-pressure transducer, Unimeasure Inc., Grants Pass, Oregon) equipped with a Hamilton valve and a P-8 adapter. Bottles were vented to atmospheric pressure after each measurement in order to avoid cumulative gas pressures beyond the response range of the P-8 adapter capable of measuring up to 50 ml of gas pressure. The pressure transducer was connected to a Fluke digital multimeter (M.F.G. Co. Inc., Mountlake Terrace, WA.) and powered by a 12 Volt solid state regulated power supply (Micronta). The multimeter response (in milliohms) was related to milliliters of gas by means of a standard curve constructed by adding known quantities of CO_2-N_2 gas to a Wheaton vial containing 100 ml of RAMM. Net gas production was calculated by subtracting gas produced in unautoclaved control bottles from that produced in test bottles.

Methane by gas chromatography (GC)

Methane production was demonstrated by injecting 5 ul of headspace gas from serum bottles into a gas chromatograph (Varian 3700, stainless steel Porapak N, 100/120 column, N_2 as carrier gas with flow rate between 30-40 ml/min) equipped with a flame ionization detector. The chromatograph was calibrated and a standard curve was constructed. Methane gas (in ml) was quantified by means of the standard curve and reported in order of degradation of the monohalogenated substrates.

Calculations

The total gas produced was divided between CO_2 and CH_4 based on the stoichiometry of the reaction which can be calculated by the Buswell equation (Tarvin and Buswell, 1934):

$$C_nH_aO_b + [n - \frac{a}{4} - \frac{b}{2}]H_2O \longrightarrow [\frac{n}{2} - \frac{a}{8} + \frac{b}{4}]CO_2 + [\frac{n}{2} + \frac{a}{8} - \frac{b}{4}]CH_4$$

For 3-IBZ, 3-ClBZ, 3-BrBZ, n=7, a=5, b=2 ==> molar fraction of CO_2 = 3.375 - 1 (corrected for one neutralized carboxyl group), of CH_4 = 3.625. Working out with 1.2 mM substrate concentration, the molar concentration of CO_2 = 2.85 mM, of CH_4 = 4.35 mM. Correcting for solubility according to Shelton and Tiedje, 1984a, the actual molar concentration of CO_2 = 0.998 mM (2.85 x .35), of CH_4 = 4.13 mM (4.35 x 0.95); theoretical CO_2 production = 22.3 ml (0.998 mM x 22.4 l/mol); theoretical CH_4 production = 92.5 ml (4.13 mM x 22.4 l/mol).

High pressure liquid chromatography (HPLC)

Samples for HPLC analysis were taken from flasks at regular time intervals, passed through a 0.45 Millipore disposable membrane filter, and stored at -10° C until analysis. Substrate depletion was monitored in a Waters Millipore system (Waters 501 HPLC pump, Novapak C_{18} column, 50% methanol/1% acetic acid as solvents , UV absorbance at 254 nm, 25 ul of sample injected into injection port). The retention times of significant peaks were compared with substrate standards (dissolved in RAMM). The area counts given by the digital integrator were then related to the concentration of external substrate standards by means of a standard curve in order to obtain substrate concentrations.

Adaptation Experiment

Five hundred ml amounts of the collected sediment were transferred using anaerobic techniques to 2-l Erlenmeyer flasks and then suspended in RAMM in a 1:2 ratio. The flasks were sealed with thick butyl rubber stoppers. The stoppers were secured in place with tape and equipped with a needle attached to Nalgene tubing trapped in a test tube filled with water to maintain atmospheric pressure in the head space of the flasks. The substrates 3-ClBZ, and 3-BrBZ were added to the sediment slurry in the flasks at concentrations in the range of 0.6 - 1.2 mM. Incubation was carried out in the dark at room temperature. Substrate disappearance was monitored by HPLC. Once total substrate depletion occurred, the same substrate (0.6 - 1.2 mM) was added again and its depletion was followed by HPLC. Strict anaerobic techniques were adhered to during the experiment. The sediment consortium was considered to be adapted after a repeated addition showed the substrate degraded without or with a much shortened lag period. Adaptation, as taken in our study, has been defined by Shimp et al. (1987) as "An increase in the ability of a microbial community to degrade a chemical after prolonged exposure to the material". Autoclaved (30 min, 10 lbs/sq. in.) sediments were incubated under the same conditions as test flasks and the breakdown also monitored by HPLC.

Cross - Adaptation Experiments

To flasks containing sediment previously adapted to a monohalogenated substrate was added polyhalogenated substrate (0.6 - 1.2 mM), 3,5-dichlorobenzoate (3,5-diClBZ), or 4-amino-3,5-dichlorobenzoate (4-amino-3,5-diClBZ). The progress of substrate disappearance was followed by HPLC.

Bacterial counts

Total bacterial numbers were determined by epifluorescence microscopy of glutaraldehyde - fixed cells which were subsequently stained with acridine orange. Moist sediment (1g) was diluted 10^{-2} in distilled water, vortexed and blended in a mini-Osterizer jar. A 10 ml sample of the suspension was fixed with pre-filtered glutaraldehyde (final concentration, 0.25%; Eastman Kodak Co.) and refrigerated. For counting, the fixed sediment suspension was further diluted 10^{-1}. A one-tenth ml was filtered through an unstained Nucleopore polycarbonate membrane (25mm membrane diameter, 0.2um pore diameter), stained with freshly prepared acridine orange (made up in 50 mM phosphate buffer, pH 7.0, to a final concentration of 0.01%; Sigma Chemical Co.) for 5 min, vacuum filtered and washed with 1.5 ml of isopropanol to reduce background staining (Geesey et al. 1978). The membrane was observed under a Reichert Neopan microscope (magnification, x1250) equipped for epifluorescence and illuminated with an HBO 50W mercury lamp (filter combination: excitation, BP 455-490; beam splitter, DS 520; barrier filters LP 418, BP515-560). Between 300 and 600 orange- and green-fluorescing bacteria were counted in 20 fields when a 10 by 10 reticule was used. Bacterial densities were estimated on sediment samples which had been refrigerated for 3 weeks and for 3 1/2 months. Counts were expressed per g dry weight. Samples of sediment were dried at 105°C to constant weight.

Viable counts

A series (0.1ml) of sediment dilutions (10^{-1} to 10^{-5}, prepared in degassed saline, 0.85%) were spread on plates of fresh brain heart infusion (BHI) agar and trypticase soy agar (TSA), both diluted 1/10, and incubated at room temperature in Scott or BBL anaerobic jars in the dark. After seven days colonies were counted, and the bacteria examined in wet mounts with a phase-contrast microscope (Zeiss GFL microscope) and in Gram stained smears.

Roll tubes

Each anaerobic Hungate type rolltube (Bellco Glass Inc., Vineland, N.J.) was amended with 0.15 g of Difco/Bacto agar (3% agar final concentration), 0.1 ml of substrate (1.2 mM) per tube, and sealed with a rubber stopper and screw-on cap (Bellco Glass Inc., Vineland, N.J.). RAMM (4.9 ml) without cysteine-Na_2S was added anaerobically to each tube while flushing with oxygen-free gas. Reducing agent (0.5 ml per tube) was added with a needle and a syringe while flushing with gas. Time was allowed for reduction to occur, after which the tubes were autoclaved at 10 lbs/sq. in. for 10 min. The autoclaved tubes were cooled to 40-50°C in a water bath, and appropriate volumes (0.1 or 1.0ml) of sediment slurry were added aseptically. The tubes were then placed in a Bellco tube spinner (Bellco Glass Inc., Vineland, N.J.) and spun, while applying ice to the tube, until agar solidified and coated the sides of the tube. The tubes were then placed in ice for 5-10 min in order to allow for further solidification of the agar medium. They were incubated in vertical position at 35-37°C in dark and checked periodically for growth.

A set of tubes was prepared as above, but the agar was allowed to solidify prior to addition of the sediment slurry. The tubes were then inoculated with ten-fold dilutions of slurry obtained from adapted flasks. Strict anaerobic technique was adhered to as in the case of preparing liquid media. Inoculated tubes were incubated in the dark at room temperature and in a 37°C incubator (Hungate, 1968).

Microscopy

Colonies growing in roll tubes were picked with a sterile Pasteur pipette, suspended in a drop of sterile water, and Gram stained. The agar drop technique, in which a drop of bacterial suspension was fused with a drop of 1% agar solution, (Hoeniger and Headley, 1969), was used for the preparation of slides for phase contrast-microscopy. The Carl Zeiss Universal Microscope (magnification, x1250) was employed to observe both Gram stained and phase-contrast slides.

RESULTS

Gas production

The effect of different concentrations was assessed by incubating all substrates at 0.3, 0.6, 1.2, and 2.4 mM. The gas production did not seem to increase significantly beyond the 1.2 mM substrate concentration. All gas production results shown are those for an initial substrate concentration of 1.2 mM (Figures 3 and 4).

Net gas production was calculated by subtracting the volume of gas produced in unautoclaved control bottles from that in test bottles. Test bottles were also compared with autoclaved control bottles. All test chemicals were degraded at both 37°C (Fig. 3) and room temperature (Fig. 4). Ethanol (positive control), BZA and 3-IBZ gave the highest gas production at both temperatures. 3-BrBZ breakdown resulted in greater gas production than both 3-ClBZ and 4-amino-3,5-diClBZ at both temperatures. At 37°C, however, 3,5-diClBZ breakdown showed slightly more gas production than both 3-BrBZ and 3-ClBZ, as well as the reference chemical cresol; however at room temperature 4-amino-3,5-diClBZ and 3,5-diClBZ showed less production.

Gas chromatography (GC)

Calibration with different volumes of CH_4 (in headspace of serum bottles containing 100 ml of RAMM) allowed for construction of a standard curve and determination of the volume of methane gas in test bottles. Methane production was tested in duplicate, and the results are shown in Table 2. In general, it was confirmed that CH_4 gas formed a certain fraction of the total gas produced in the test bottles. The same pattern as that observed with gas production was revealed: the greatest volume of methane was produced in bottles amended with 3-IBZ, followed by 3-BrBZ and 3-ClBZ (smallest volume).

High pressure liquid chromatography (HPLC)

Preliminary HPLC analyses of substrate disappearance from sediments at both sites revealed that the test compounds were degraded in the same order as that indicated by gas production and GC analysis: 3-IBZ (most rapid), 3-BrBZ, and 3-ClBZ. A transient formation of benzoic acid, a metabolic intermediate, was also noted. In general, the concentration of benzoic acid first increased and then decreased as substrates became depleted. It was further noted that small amounts of intermediates, other than benzoic acid accumulated.

Adaptation experiment

Adaptation experiments were conducted with regard to 3-ClBZ and 3-BrBZ on sediments from all sites. HPLC analysis revealed that 3-BrBZ was degraded more rapidly than 3-ClBZ (average time for complete degradation of the substrate was 7 weeks for 3-BrBZ and 15 weeks for 3-ClBZ, Table 4). Lag periods prior to the onset of degradation were found to be 2.5 weeks for 3-BrBZ and 11.5 weeks for 3-ClBZ (Table 3). Subsequent additions of these substrates to the same sediment resulted in immediate degradation, with much shortened lag period (Figures 5 to 10). Substrates were depleted in a much shorter time, averaging from 4 weeks for 3-BrBZ to 6 weeks for 3-ClBZ (Table 5).

Cross - adaptation experiment

Organisms adapted to 3-BrBZ after successive addition of 3-BrBZ were able to completely deplete 0.6-1.2mM of 3,5-diClBZ within 3 weeks. Organisms acclimated to 3-ClBZ required 4 to 5 weeks for 3,5-diClBZ depletion. The amino derivative, 4-amino-3,5-diClBZ was , on the average, only partially degraded by organisms adapted to 3-ClBZ even after 16 weeks. Organisms adapted to 3-BrBZ, however, were able to completely degrade 4-amino-3,5-diClBZ within 6 weeks (Table 6 and 7, Figures 5 to 10).

Bacterial density and viable organisms in sediment

Total counts of bacteria did not change significantly during prolonged storage ($P < 0.001$, Table 8). Densities in the range of 1.2 to 1.5×10^{10}/g (dry weight) were higher than those found in nonpolluted sediments of most lakes, i.e. 5×10^8 to 2×10^9/g (dry weight) (Hoeniger, 1985; Jones, 1982). The viable counts (as colony-forming units, CFU) of sediment anaerobes (Table 9) were fifty thousand-fold lower than the total counts (Table 8), as expected. The bacteria were mainly small Gram-negative rods with some larger Gram-positive bacilli.

Growth on Roll tubes

Tubes inoculated after agar solidification and incubated at 37°C exhibited growth (transparent colonies, approximately 0.1mm in diameter) after 2 days for the three monohalogenated substrates (3-IBZ, 3-ClBZ, 3-BrBZ). Tubes inoculated after agar solidification and incubated at room temperature showed growth (transparent colonies, approximately 0.1mm in diameter) after three to six weeks (for all three substrates). Tubes in which slurry was incorporated into agar prior to solidification were incubated at 37°C and showed numerous growth forms after two weeks of incubation (Table 10).

Microscopy

Gram staining of smears obtained from roll tubes, and of suspensions obtained from flasks and serum bottles, revealed Gram negative rods shown by phase-contrast to range from 1.4 to 5.8 um in length and 0.5 to 0.7 um in width. A few of the shorter rods contained bipolar granules. A number of Gram negative cocci (<0.6 um in diameter) and Gram positive cocci (0.6 um in diameter) were also observed. Phase-contrast microscopy of agar drop preparations (Figures 11 to 16) revealed rods ranging from 1.3 to 6.5 um in length and 0.6 um in width. Some rods contained numerous granules ranging from 3 to 6 per cell. A few rods with terminal spores were slightly curved and arranged in pairs. In general, the observed organisms were representative of a typical fresh-water consortium.

DISCUSSION AND CONCLUSIONS

The results of this research study showed that halogenated benzoates, simple and more complex, were biodegradable in sediments removed from sites along the Toronto waterfront. It was further shown that the anaerobic consortia responsible for breakdown of compounds were able to cross-adapt and degrade polyhalogenated pollutants. The results suggest that cross-adaptation may be an effective approach to a more rapid biodegradation of complex pollutants in lake sediments.

The test compounds were degraded to varying degrees by sediment cultures (Figures 3 and 4). As expected, ethanol (positive control) and BZA (reaction intermediate) breakdown resulted in highest gas production and demonstrated the capability of the sediment system for anaerobic biodegradation. No apparent lag periods or gas production were observed, in contrast to those reported by Shelton and Tiedje (1984a). This could be accounted for by increased background gas production in test bottles. The gas production from 3-ClBZ and 4-amino-3,5-diClBZ was low as compared to that from 3-BrBZ. Horowitz et al., 1982, reported that 4-amino-3,5-diClBZ is transformed to 4-amino-3-ClBZ without significant mineralization. This complies with the low values of gas production observed for the amino derivative. Very low levels of gas production were detected in autoclaved controls, emphasizing that the mineralization process observed was of a biological nature. Horowitz et al., 1982, further found that the dehalogenation reaction was inhibited at temperatures well above 39°C, thus "supporting the contention that dehalogenation is a biologically catalyzed reaction". Methane production from the three monohalogenated compounds was confirmed by GC (Table 2), with greatest production obtained from breakdown of 3-IBZ and the lowest from that of 3-ClBZ, this pattern being the same as that found with gas production.

Depletion of substrates as determined by HPLC revealed a particular pattern with regard to their use by sediment cultures. The organisms preferred 3-IBZ to 3-BrBZ and 3-ClBZ. Benzoic acid was produced as a biodegradation intermediate, in agreement with other studies (Suflita et al, 1982, 1983; Horowitz et al, 1983) which have shown that benzoic acid is the intermediate in reductive dehalogenation. These authors further suggested that the removal of ring-substituted halogens is the initial step in the anaerobic degradation process and must occur prior to the opening of the aromatic ring. Presumably a complete dehalogenation is required before a substrate can be mineralized to CH_4 and CO_2. Dehalogenation has been shown to be exergonic, yielding biologically useful energy, used for growth by the species responsible for dehalogenation (Dolfig and Tiedje, 1987). Thus in anaerobic habitats, microorganisms thriving on anaerobic degradation of halogenated aromatic xenobiotics may have a natural advantage over organisms which do not utilize the energy yielded in this particular reaction.

The ability of sediment microorganisms to adapt and cross-adapt to halogenated compounds would naturally be of great interest. The adaptation and cross-adaptation experiments provided some insight in this direction. There was a lag period required before substrate depletion occurred for the monohalogenated benzoates (Table 3). However, once the sediment organisms were adapted to a monohalogenated compound, further additions of the same substrate resulted in immediate depletion (Table 5). Some possible ways of accounting for this result may be an increase in microbial numbers of specific degraders (Aelion et al., 1987, Shimp et al., 1987, Wiggins et al., 1987), or perhaps the induction of specific enzymes (Horowitz et al., 1983) or an increased activity of non- specific dehalogenating enzymes as a consequence of the greater numbers of degraders selected out in the population. The cross-adaptation results showed that both

3,5-diClBZ and 4-amino-3,5-diClBZ were degraded more rapidly in sediments previously adapted to 3-BrBZ than in those adapted to 3-ClBZ (Table 6 and 7). Thus, pre-adaptation to 3-BrBZ may be an effective measure in inducing breakdown of the dichloro derivatives. As expected, in autoclaved control flasks, substrate depletion did not occur, further adding to the evidence for biodegradation as a biological process. Horowitz and colleagues (1983) also showed that "dehalogenation activity was inhibited in acclimated sediment by autoclaving, gamma irradiation, or formaldehyde treatment relative to non-sterile sediment".

The degradative process is a probable result of cometabolism of different species existing in a mutualistic relationship in the sediment. This is often the case with bacterial populations involved in methane production (Atlas and Bartha, 1981). The presence of various microbial forms and their viability points to a cometabolizing community, or a consortium. For example, Shelton and Tiedje (1984b) characterized the bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoate. They described short Gram negative rods (Syntropus buswellii), a benzoate oxidizing bacterium, slightly curved, long, Gram negative rods (Methanogen hungatei), irregular rods with various inclusions (butyrate oxidizers), and long, thin rods (other methanogens) (Tiedje et al, 1986). A consortium may contain other major groups of obligate anaerobes, such as Clostridia (fermenters), Desulfovibrio or Desulphotomaculum species (sulphate reducers), proton reducers (oxidize fatty acids and alcohols to acetate and CO_2), and CO_2 reducers (use CO_2 as a terminal electron acceptor and create CH_4 or acetate) (Kaspar and Tiedje, 1982).

In summary, our results imply that (i) in order of preference, 3-iodobenzoic acid, 3-bromobenzoic acid, and 3-chlorobenzoic acid can be degraded to CH_4 and CO_2 by Lake Ontario sediment organisms under anaerobic conditions; (ii) the biodegradation is accomplished under our test conditions by an anaerobic consortium of microorganisms characterized by a high proportion of Gram negative rods; (iii) the biodegradation probably proceeds via a reductive dehalogenation pathway with benzoic acid as a main intermediate; (iv) cross-adaptation can lead to the degradation of more complex substrates with 3-BrBZ shown in this study as the preferred "adaptation" substrate.

The next step should be to isolate and cultivate the consortium in order to allow for controlled use of such a cometabolizing community in waste water facilities. This approach would provide an almost natural mode for the biodegradation of industrial wastes prior to their release into our lake ecosystems.

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Table 1. Sediment sampling sites along the Toronto Waterfront and major sources of pollution (adapted from Persaud et al., 1985).

Site	Location		Sand %	Loss on ignition %	Total PCB ng/g	Solvent extractables ug/g
332	Humber Bay	Humber Bay STP*	7.1	8.2[e]	700[e]	13415[e]
335	Humber Bay	Humber Bay STP	1.7	89.6[e]	690[e]	3550[e]
1355	Toronto Harbour	oil, grease**,	-	6.6[e]	120[e]	6850[e]
1375	Don River Mouth	Don River		6.6[e]	-	4660[e]
A	Ashbridges Bay	Main STP	-	-	-	-
2216	Eastern Beaches	Main STP	97.3	0.2	<20	20
MOE GUIDELINES				6.0	50	1500

* STP = sewage treatment plant

[e] level exceeds Ministry of the Environment (MOE) guidelines

** mainly in boat slips from "loss on ignition"

Other sources of pollution at sampling sites are agricultural and urban runoff. The contaminants may be grouped as organics (volatiles, oil, grease, PCB's), nutrients (phosphorus, nitrogen), and trace metals (copper, lead, mercury). Note that all sites except for 2216 have elevated levels of organic contaminants. Site A was also located in a highly contaminated area.

Table 2. Production of methane as determined by gas chromatography, also expressed as percentage of theoretical gas production

Site	substrate	incubation time (weeks)	volume of methane (ml)	methane as percentage of theoretical methane production
1375	3-IBZ	7	46.0	49.7
1375	3-IBZ	7	42.3	45.7
		average:	44.2	47.7
1375	3-BrBZ	7	34.1	36.8
1375	3-BrBZ	7	40.6	43.9
		average:	37.4	40.4
1375	3-ClBZ	7	6.3	6.8
1375	3-ClBZ	7	5.2	5.7
		average:	5.8	6.3

Using the Buswell equation, theoretical methane production, based on total degradation of 1.2 mM of substrate to methane and carbon dioxide, was found to be 92.5 ml (100%). Note that the greatest methane volume was produced from 3-IBZ and the smallest volume from 3-ClBZ, following the pattern of both gas production and substrate degradation.

Table 3. Lag period required for adaptation to monohalogenated substrates in anoxic sediments

Site	Substrate			
	3-chlorobenzoate		3-bromobenzoate	
	Time (weeks)	Average time	Time (weeks)	Average time
332	14		4	
335	13		4	
1355	13	11.5	2	2.5
1375	8		2	
A	9		<1	
2216	12		9 (oxidized)*	

Substrate disappearance was monitored by HPLC. Initial substrate concentration was in the range of 0.6 to 1.2mM/l. Note markedly shortened lag period observed for 3-BrBZ.

*This value was not used in calculations of average lag period. The sediment from this site most probably became oxidized due to its high content of inorganic materials (97.3% sand) and thus inability to support microbial growth.

Table 4. Time required for complete degradation of monohalogenated substrates (adaptation) in anoxic sediments

Site	Substrate			
	3-chlorobenzoate		3-bromobenzoate	
	Time (weeks)	Average time	Time (weeks)	Average time
332	16		9	
335	15		9	
1355	15	15	6	7
1375	14		5	
A	16		7	
2216	14		10*	

Substrate disappearance was monitored by HPLC. Initial substrate concentration was in the range of 0.6 - 1.2 mM. Note markedly shortened times needed for breakdown of 3-BrBZ.

* Oxidation began to show after 8 weeks of incubation. This value was not used in calculations of average time for complete degradation.

Table 5. Time required for subsequent complete degradation of monohalogenated substrates following initial adaptation

Site	Substrate			
	3-chlorobenzoate		3-bromobenzoate	
	Time (weeks)	Average time	Time (weeks)	Average time
332	5		3	
335	5		3	
1355	6	6	3	4
1375	7		4	
A	7		5	
2216	8		oxidized	

Substrate disappearance was monitored by HPLC. Substrates (0.6 - 1.2 mM) were added immediately after initial substrate depletion. The cross-adaptation was again shorter with 3-BrBZ, but the difference was not as obvious as for initial adaptation.

Table 6. Time required for complete degradation of polyhalogenated substrates (cross-adaptation) in sediments adapted to monohalogenated substrates

Cross-adapted to:	3,5-dichlorobenzoate		4-amino-3,5-dichlorobenzoate	
Site	Initially adapted to:			
	3-chlorobenzoate	3-bromobenzoate	3-chlorobenzoate	3-bromobenzoate
	Time (weeks)			
332			N/D[a]	12
335	5	3		
1355			N/D[a]	6
1375	4	3		
A			12	7
2216			N/D[a]	oxidized

N/D[a] Substrate was not degraded even after 16 weeks.

Substrate (0.6 - 1.2 mM, initial concentration) disappearance was monitored by HPLC. Sediments were initially adapted to a monohalogenated substrate. Greater degradation of the dichloro derivatives was achieved in sediments initially adapted to 3-bromobenzoate than in those initialy adapted to 3-chlorobenzoate.

Table 7. Comparative depletions of substrates following adaptation and cross-adaptation

substrate	3-BrBZ	3-ClBZ
initial	7 weeks	15 weeks
subsequent (adaptation)	4 weeks	6 weeks
cross-adaptation to:		
3,5-diClBZ	3 weeks	4.5 weeks
4-amino-3,5-diClBZ	6 weeks	12+

Note that degradation times in the case of 3-ClBZ are much longer than those for 3-BrBZ.

Table 8. Total bacterial counts by epifluorescence microscopy of Toronto Harbour sediment.

Storage time (4°C)	date of counting		cell counts per g (dry weight)
3 weeks	Dec. 12/1985		$0.874x10^{10}$
			$0.951x10^{10}$
	Jan. 3/1986		$1.494x10^{10}$
			$1.444x10^{10}$
		mean	$1.19\ x10^{10}$
10 weeks	Mar. 6/1986		$1.502x10^{10}$
			$1.505x10^{10}$
		mean	$1.50\ x10^{10}$

Total bacterial counts did not change significantly during prolonged storage ($P < 0.001$). Densities in the range of 1.2 to $1.5x10^{10}$/g (dry weight) are higher than found in nonpolluted sediments of most lakes.

Table 9. Viable counts of sediment anaerobes plated on two solid media and incubated 7 days at room temperature.

type of medium	colony-forming units (CFU's)/g (dry weight)
brain heart infusion agar	$4.5x10^{5}$
trypticase soy agar	$2.9x10^{5}$

The viable counts were four to five orders of magnitude lower than the total counts (Table 8), as expected.

Table 10. Bacterial growth on roll tubes with sediment incorporated into agar prior to solidification and incubated at 37°C.

site	substrate	volume of inoculum	description
332	3-BrBZ	0.1 ml	large white colonies, 3mm in dia., no distinct edge
		1.0 ml	small white colonies, pin point black colonies
	3-ClBZ	1.0 ml	large white colonies
		1.0 ml	large white colonies, pin point black colonies
335	3-BrBZ	1.0 ml	small white colonies, pin point black colonies
	3-ClBZ	1.0 ml	pin point black colonies
		1.0 ml	small black colonies
1355	3-BrBZ	0.1 ml	small black, small white colonies
		1.0 ml	small black colonies
		1.0 ml	pin point black colonies
	3-ClBZ	0.1 ml	small black colonies
		1.0 ml	small grey colonies
		1.0 ml	few (2) small white colonies, small (<0.5mm) black colonies
1375	3-BrBZ	1.0 ml	all black colonies
		1.0 ml	small black colonies
	3-ClBZ	0.1 ml	small black colonies
		1.0 ml	small black colonies
		1.0 ml	small black & white colonies
2216	3-BrBZ	1.0 ml	small black colonies
	3-ClBZ	0.1 ml	small black colonies
		1.0 ml	small black colonies
		1.0 ml	small black colonies
A	3-BrBz	0.1 ml	small black colonies
A	3-BrBZ	1.0 ml	black and smaller white colonies
		1.0 ml	large white (2-3mm) colonies, pin point black colonies
	3-ClBZ	0.1 ml	large white colonies
		1.0 ml	large mucoid white and small black colonies

Bacterial growth was produced in all roll tubes. A variety of numerous colonies was observed, ranging from large mucoid white colonies to small black colonies. Note that sediment used for preparation of inoculating slurry was collected on October 2, 1987.

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Figure 1C. Sediment sampling sites in the Toronto Harbour (1355, 1375) (reproduced from Persaud et al., 1985).

Figure 1D. Sediment sampling sites in the East Headland area (A) (reproduced from Persaud et al., 1985).

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Figure 6. Biodegradation of substrates in sediment culture from site 332, Humber Bay, as monitored by HPLC.

Figure 7. Biodegradation of substrates in sediment culture from site 335, Humber Bay, as monitored by HPLC.

Figure 8. Biodegradation of substrates in sediment culture from site 1355, Toronto Harbour, as monitored by HPLC.

Figure 9. Biodegradation of substrates in sediment culture from site 1375, Toronto Harbour, as monitored by HPLC.

Figure 10. Biodegradation of substrates in sediment culture from site 2216, Eastern Beaches, as monitored by HPLC.

Figure 11. Some microbial forms as observed by phase-contrast microscopy.

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Figure 1B. Sediment sampling sites in the Humber Bay (332, 335) (reproduced from Persaud et al., 1985).

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Figure 1D. Sediment sampling sites in the East Headland area (A) (reproduced from Persaud et al., 1985).

Figure 1E. Sediment sampling sites in the Eastern Beaches (2216) (reproduced from Persaud et al., 1985).

Figure 2. Hungate apparatus used for preparation of all oxygen-free media, substrates and sediment slurry.
(1) Nupro valves for fine metering
(2) Fume hood
(3) Bracket to hold furnace
(4) Furnace - capable of heating to 350°C
(5) Glass Column packed with copper fillings
(6) Rotameter capable of delivering at least 1.5 litres per minute of gas
(7) 2 c.c. glass syringe with luer-lok tip with a modified end to fit into butyl rubber, and packed with cotton.
(8) 17 guage hypodermic needle, 7" long, with tip filed off
(9) Butyl rubber tubing

Figure 3. Cumulative net gas production from sediment cultures collected at site 332, Humber Bay, and incubated at 37°C. Initial substrate concentration was in the range of 1.2 mM. Note the insignificant amount of gas produced in autoclaved control bottles, the low gas production in bottles with the amino derivative, and 3-ClBZ, and high gas production in bottles with BZA, 3-IBZ and ethanol.

Figure 4. Cumulative net gas production from sediment cultures collected at site 332, Humber Bay, and incubated at room temperature. Initial substrate concentration was 1.2 mM. Note the insignificant amount of gas produced in autoclaved control bottles and the low gas production in bottles with the two di-chloro compounds and 3-ClBZ, and high gas production in bottles with BZA, 3-IBZ and ethanol.

Figure 5. Biodegradation of substrates in sediment culture from site A, Ashbridges Bay, as monitored by HPLC.
A.●: initial breakdown of 3-ClBZ (note the long lag period)
  ▲: breakdown of 3-ClBZ after subsequent addition of substrate
  ○: cross-adaptation to 4-amino-3,5-diClBZ
B.●: initial breakdown of 3-BrBZ
  ▲: breakdown of 3-BrBZ after subsequent addition of substrate
  ○: cross-adaptation to 4-amino-3,5-diClBZ

Figure 6. Biodegradation of substrates in sediment culture from site 332, Humber Bay, as monitored by HPLC.
A.●: initial breakdown of 3-ClBZ (note the long lag period)
Δ: breakdown of 3-ClBZ after subsequent addition of substrate
○: cross-adaptation to 4-amino-3,5-diClBZ (note that the substrate was not depleted even after 16 weeks)
B.●: initial breakdown of 3-BrBZ
Δ: breakdown of 3-BrBZ after subsequent addition of substrate
○: cross-adaptation to 4-amino-3,5-diClBZ

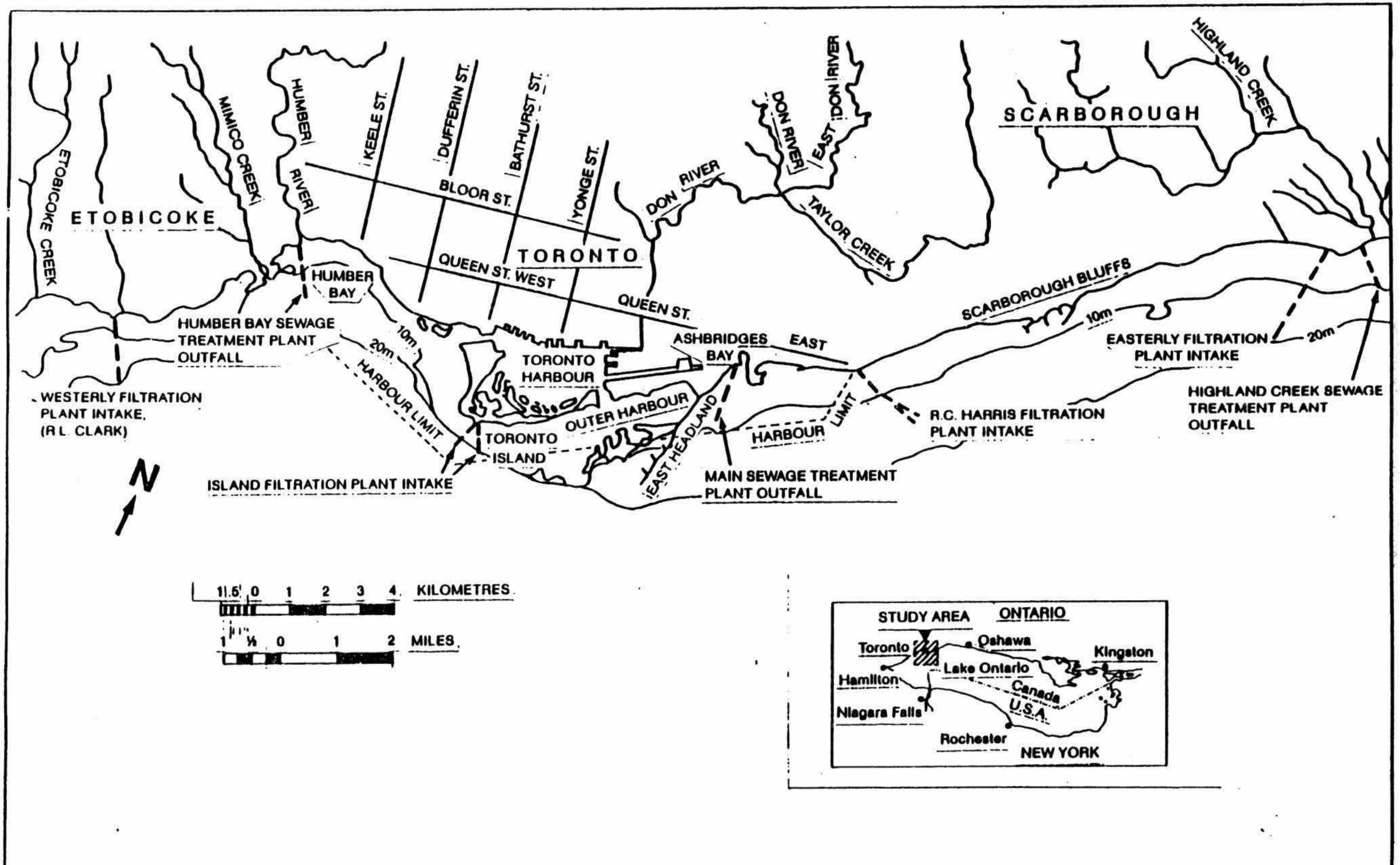
Figure 7. Biodegradation of substrates in sediment culture from site 335, Humber, Bay as monitored by HPLC.
A.●: initial breakdown of 3-ClBZ (note the long lag period)
Δ: breakdown of 3-ClBZ after subsequent addition of substrate
○: cross-adaptation to 3,5-diClBZ
B.●: initial breakdown of 3-BrBZ
▲: breakdown of 3-BrBZ after subsequent addition of substrate
○: cross-adaptation to 3,5-diClBZ

Figure 8. Biodegradation of substrates in sediment culture from site 1355, Toronto Harbour, as monitored by HPLC.
A.●: initial breakdown of 3-ClBZ (note the long lag period)
Δ: breakdown of 3-ClBZ after subsequent addition of substrate
○: cross-adaptation to 4-amino-3,5-diClBZ (note that the substrate was not depleted even after 16 weeks)
B.●: initial breakdown of 3-BrBZ
Δ: breakdown of 3-BrBZ after subsequent addition of substrate
○: cross-adaptation to 4-amino-3,5-diClBZ

Figure 9. Biodegradation of substrates in sediment culture from site 1375, Toronto Harbour, as monitored by HPLC.
A.●: initial breakdown of 3-ClBZ (note the long lag period)
Δ: breakdown of 3-ClBZ after subsequent addition of substrate
○: cross-adaptation to 3,5-diClBZ
B.●: initial breakdown of 3-BrBZ
Δ: breakdown of 3-BrBZ after subsequent addition of substrate
○: cross-adaptation to 3,5-diClBZ

Figure 10. Biodegradation of substrates in sediment culture from site 2216, Eastern Beaches, as monitored by HPLC.
A.●: initial breakdown of 3-ClBZ (note the long lag period)
Δ: breakdown of 3-ClBZ after subsequent addition of substrate
○: cross-adaptation to 4-amino-3,5-diClBZ (note that the substrate was not degraded even after 16 weeks)
B. The sediment culture became oxidized after 12 weeks of incubation.
No further measurements were carried out in the oxidized culture.
●: initial breakdown of 3-BrBZ

Figure 11. Some microbial forms as observed by phase-contrast microscopy: typical Gram-negative rods (A), chains of Gram-positive cocci (B), filamentous forms (C), Gram-negative rods and cocci (D). Bar represents 10 um.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TORONTO
QUEEN ST. WEST
QUEEN ST.
DON RIVER
EAST DON RIVER
TAYLOR CREEK
SCARBOROUGH
HIGHLAND CREEK
HUMBER BAY
HUMBER BAY SEWAGE TREATMENT PLANT OUTFALL
10m
20m
HARBOUR LIMIT
WESTERLY FILTRATION PLANT INTAKE, (R.L. CLARK)
TORONTO HARBOUR
OUTER HARBOUR
TORONTO ISLAND
ISLAND FILTRATION PLANT INTAKE
ASHBRIDGES BAY
EAST
EAST HEADLAND
MAIN SEWAGE TREATMENT PLANT OUTFALL
HARBOUR LIMIT
R.C. HARRIS FILTRATION PLANT INTAKE
SCARBOROUGH BLUFFS
10m
20m
EASTERLY FILTRATION PLANT INTAKE
HIGHLAND CREEK SEWAGE TREATMENT PLANT OUTFALL
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1 ½ 0 1 2 MILES
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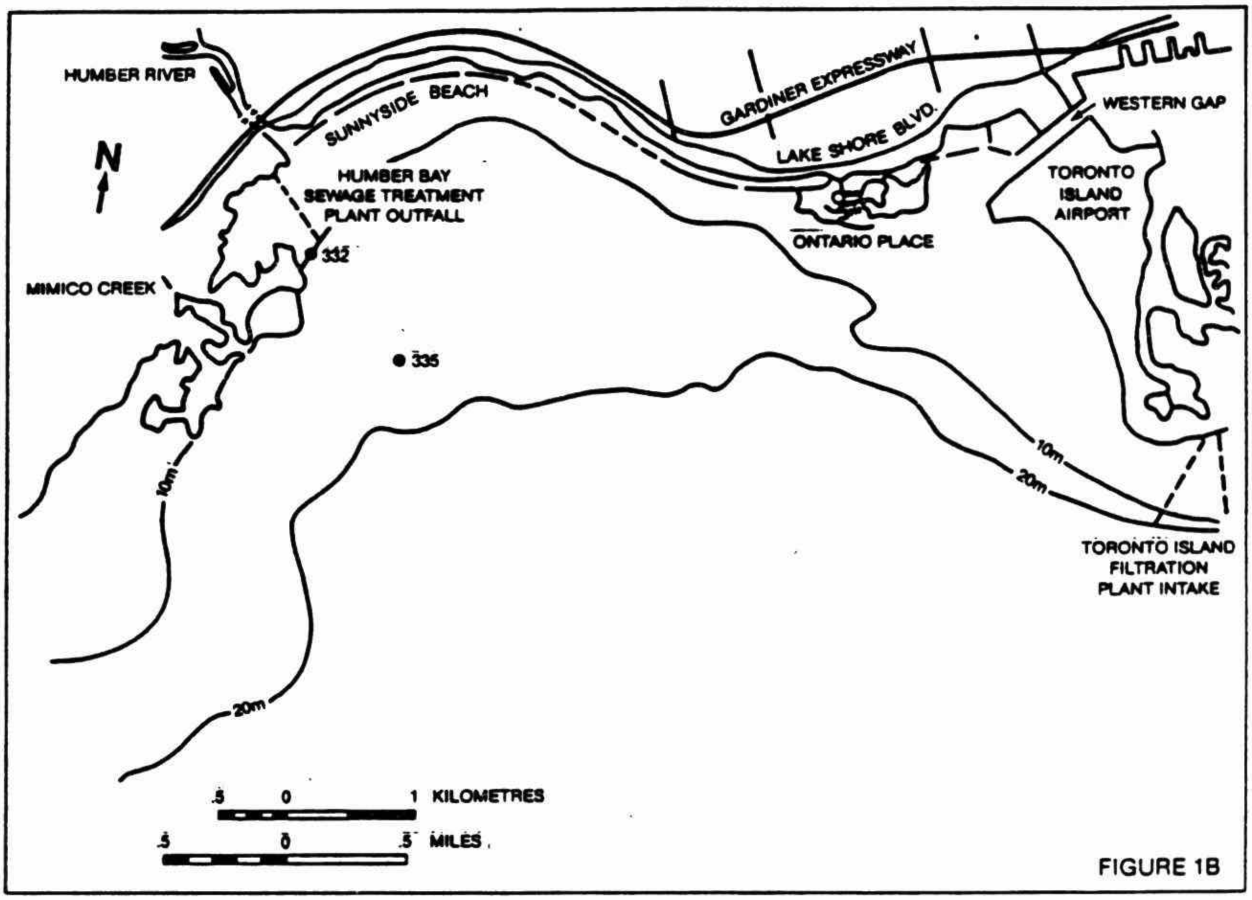
FIGURE 1A



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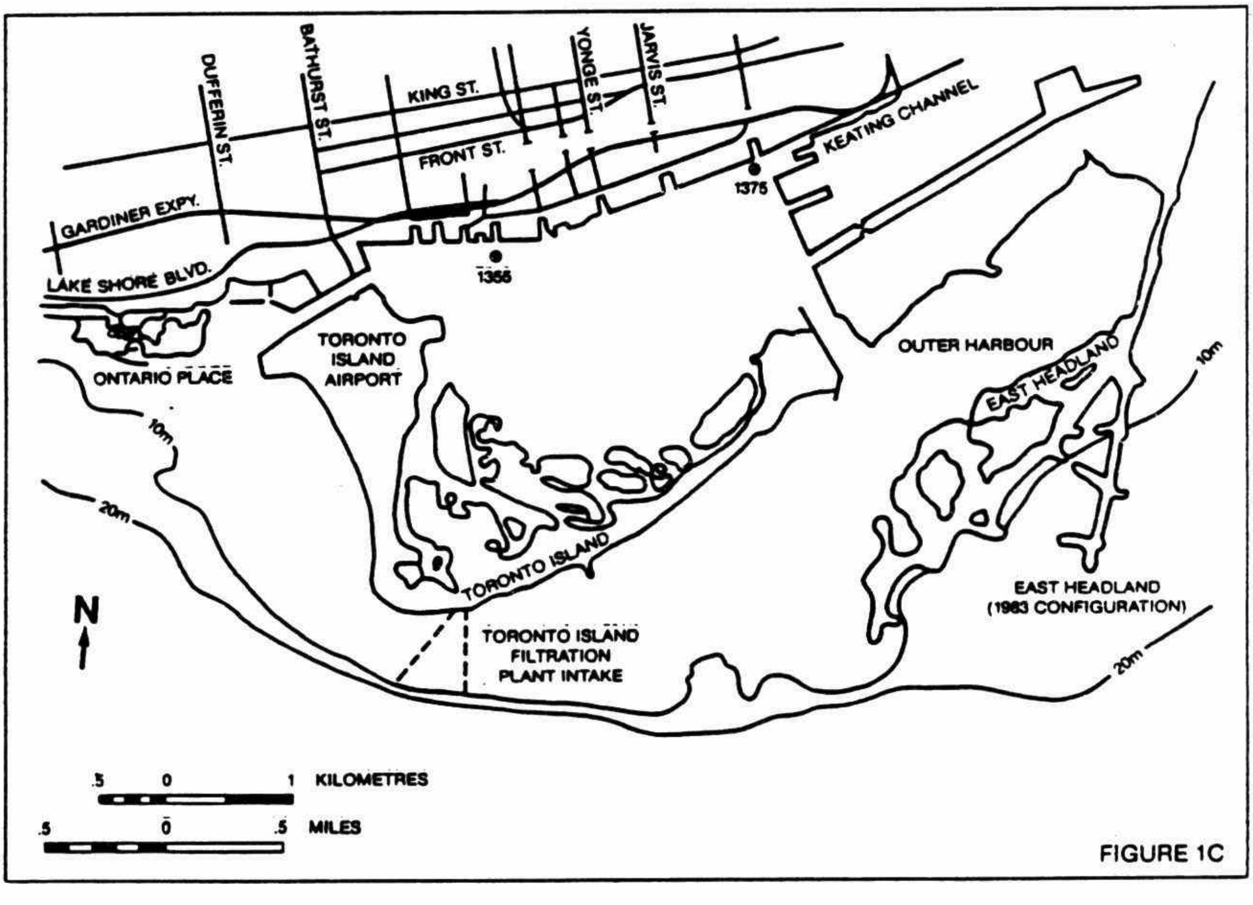

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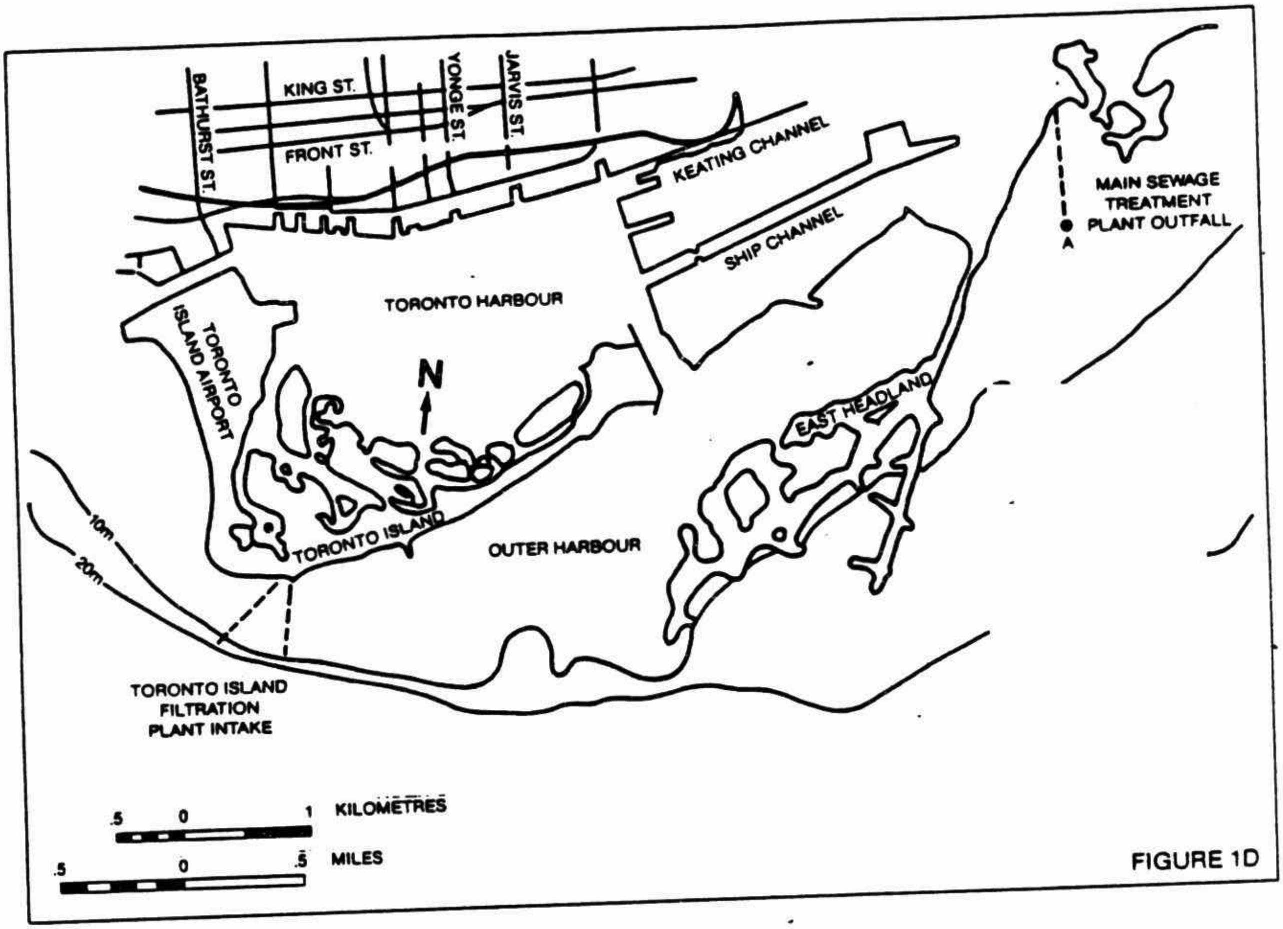

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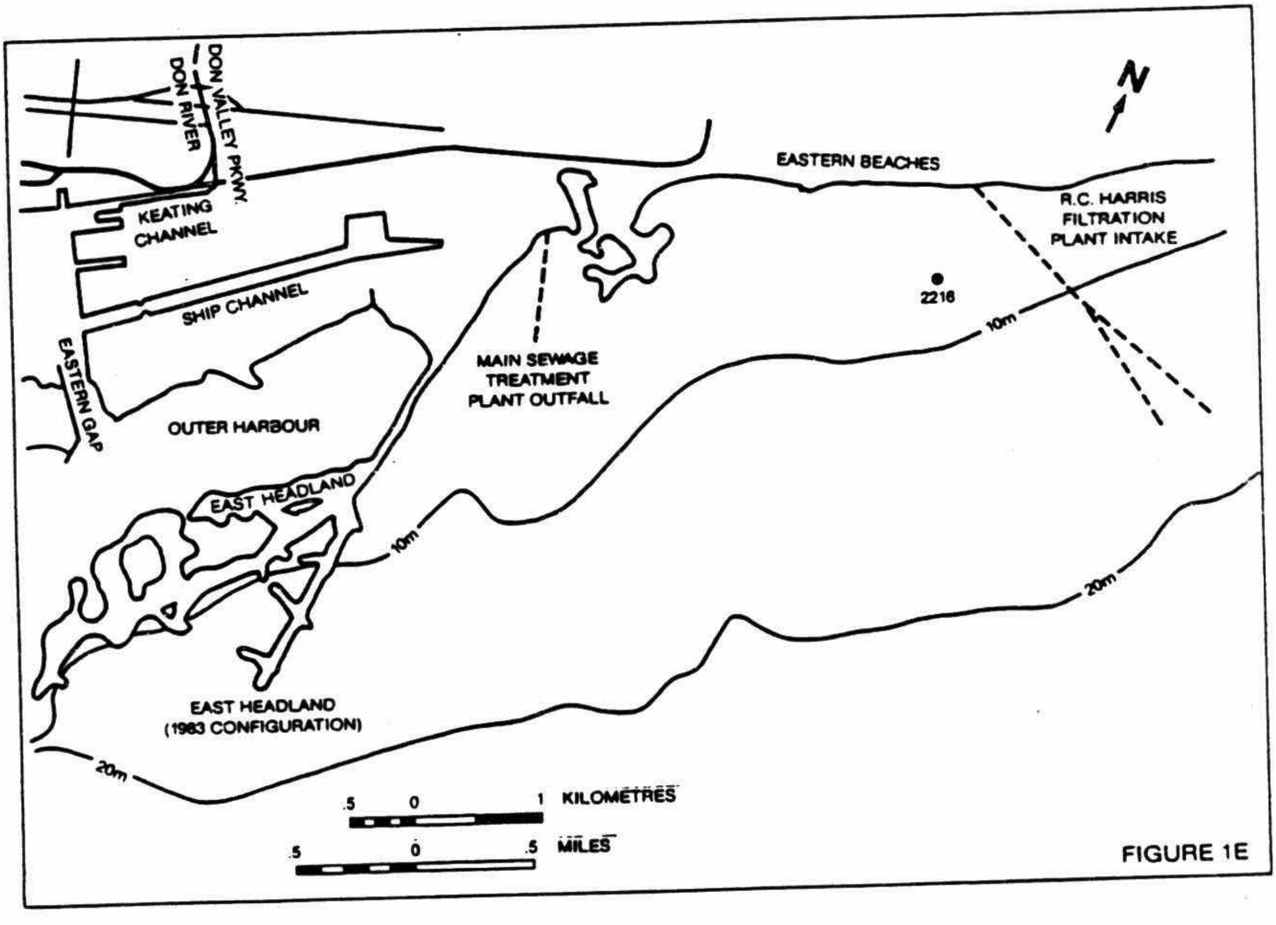

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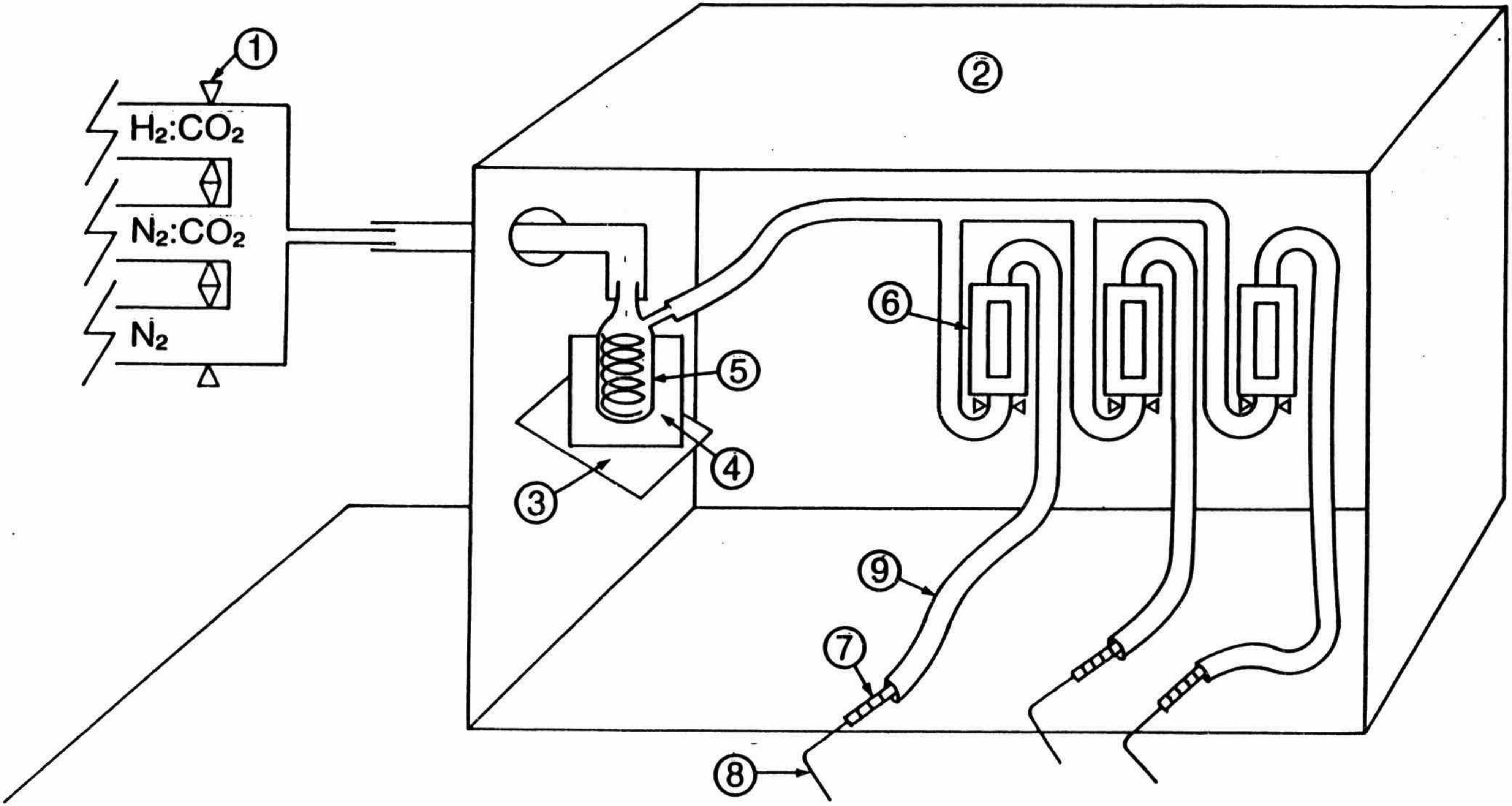

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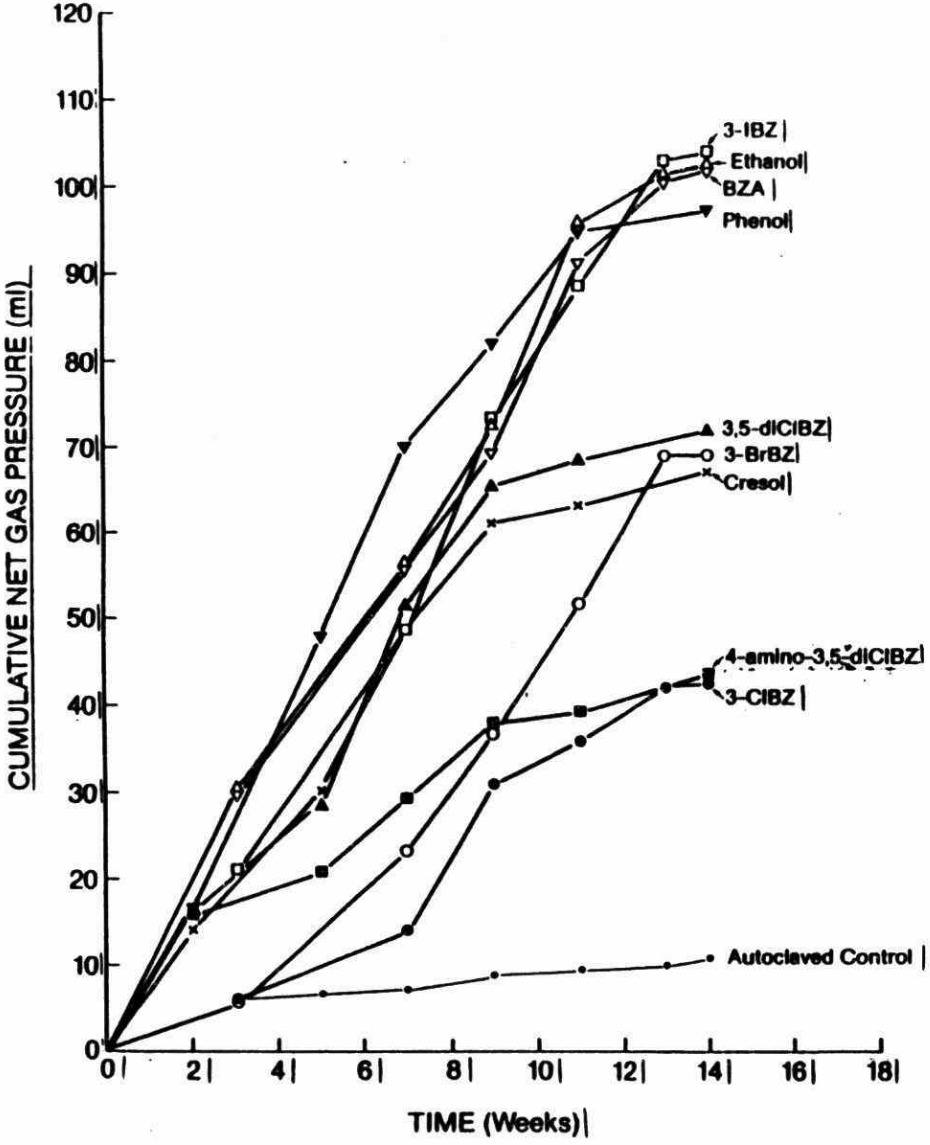

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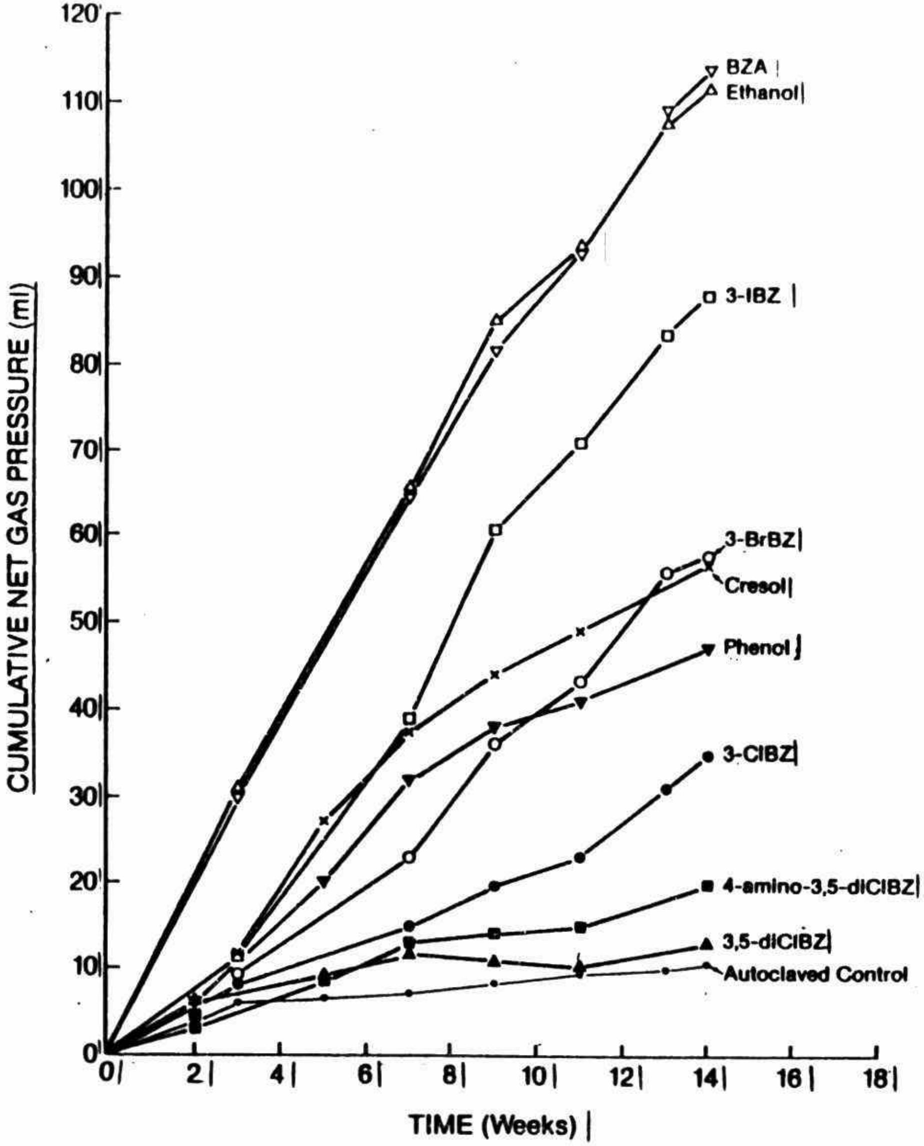

FIGURE 4

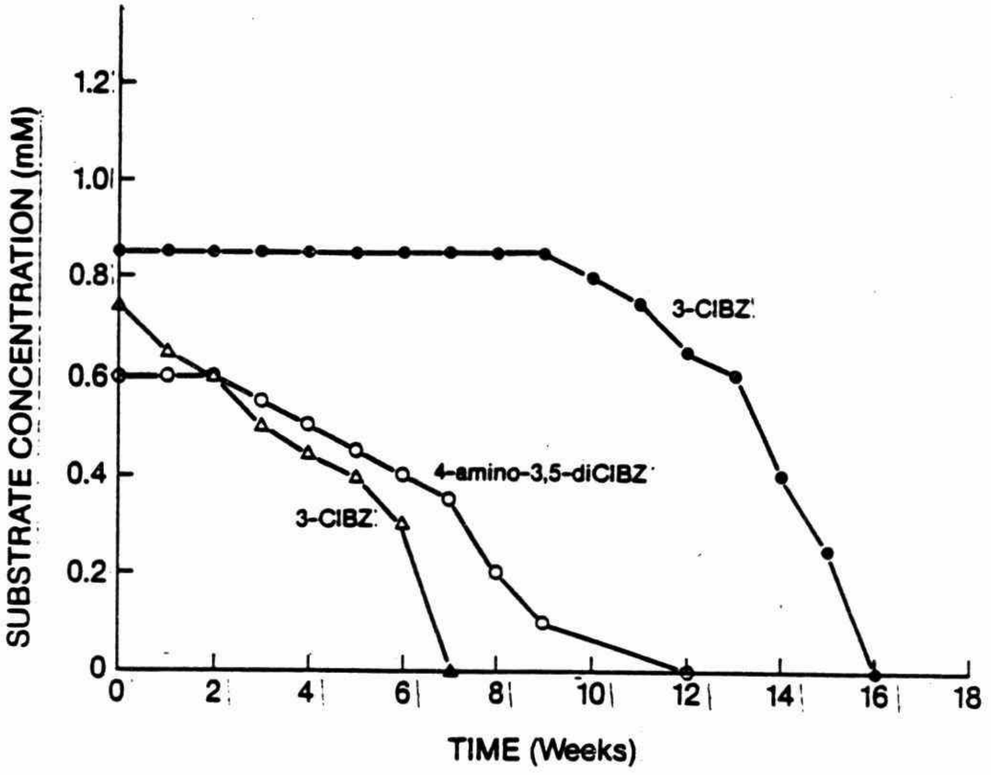

FIGURE 5A

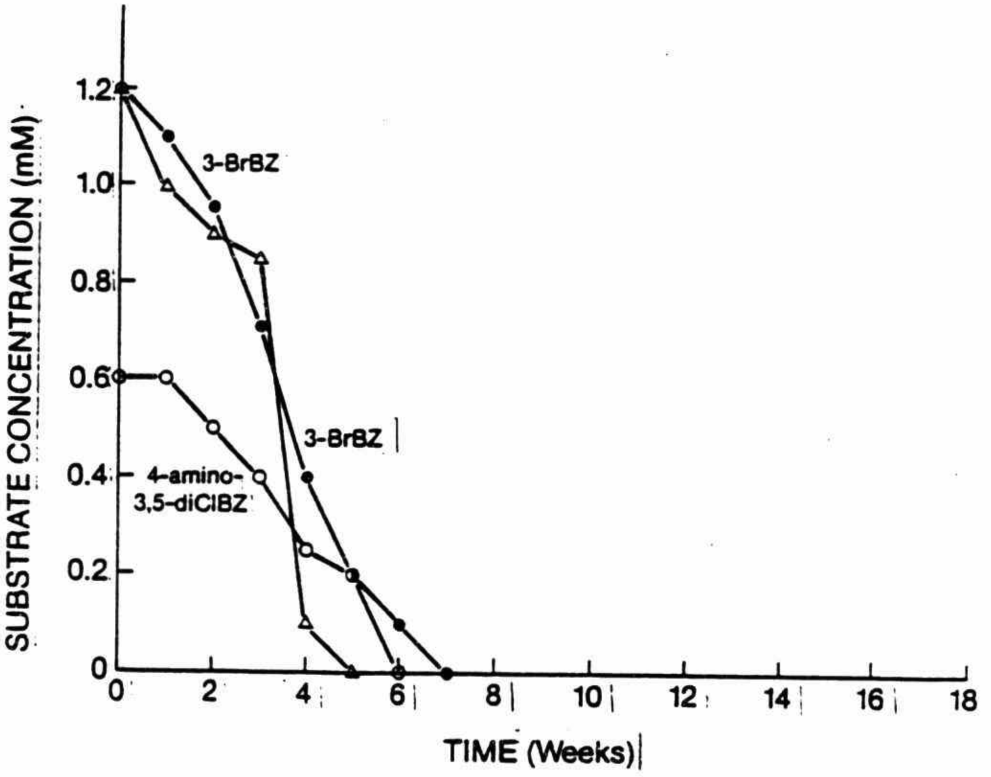

FIGURE 5B

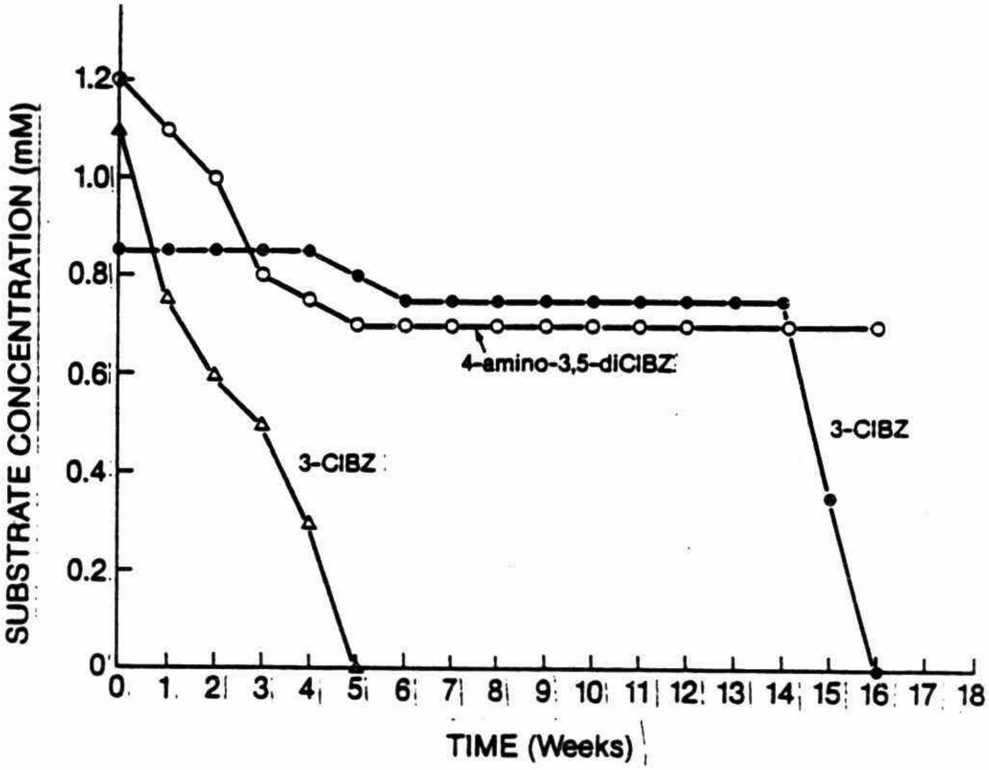

FIGURE 6A

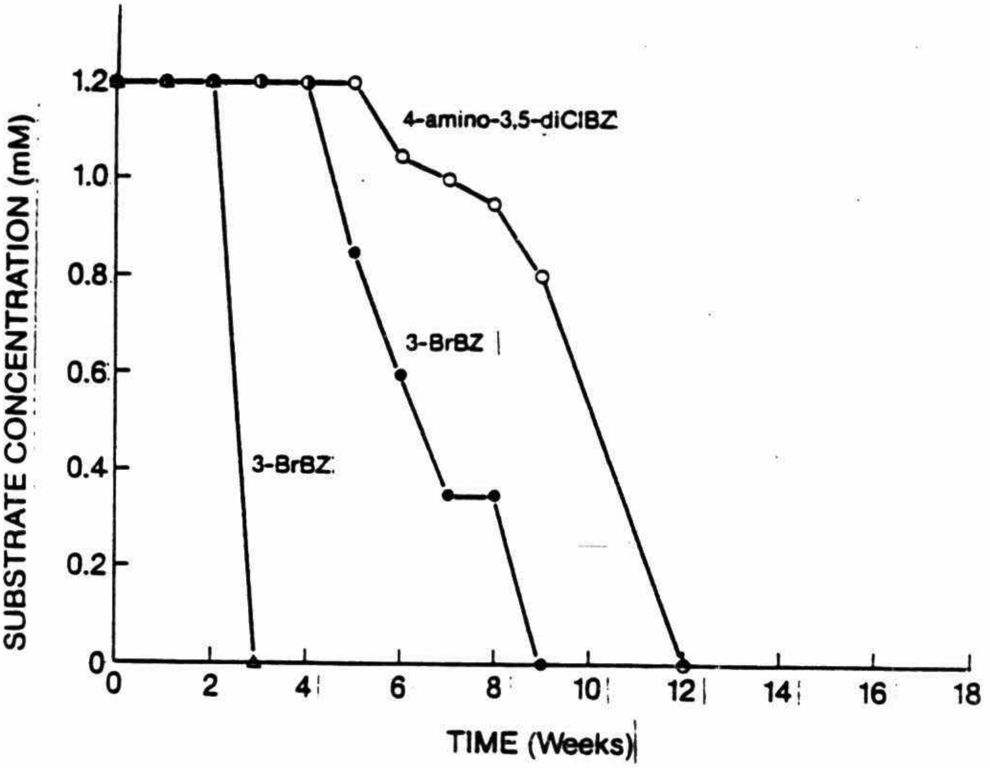

FIGURE 6B

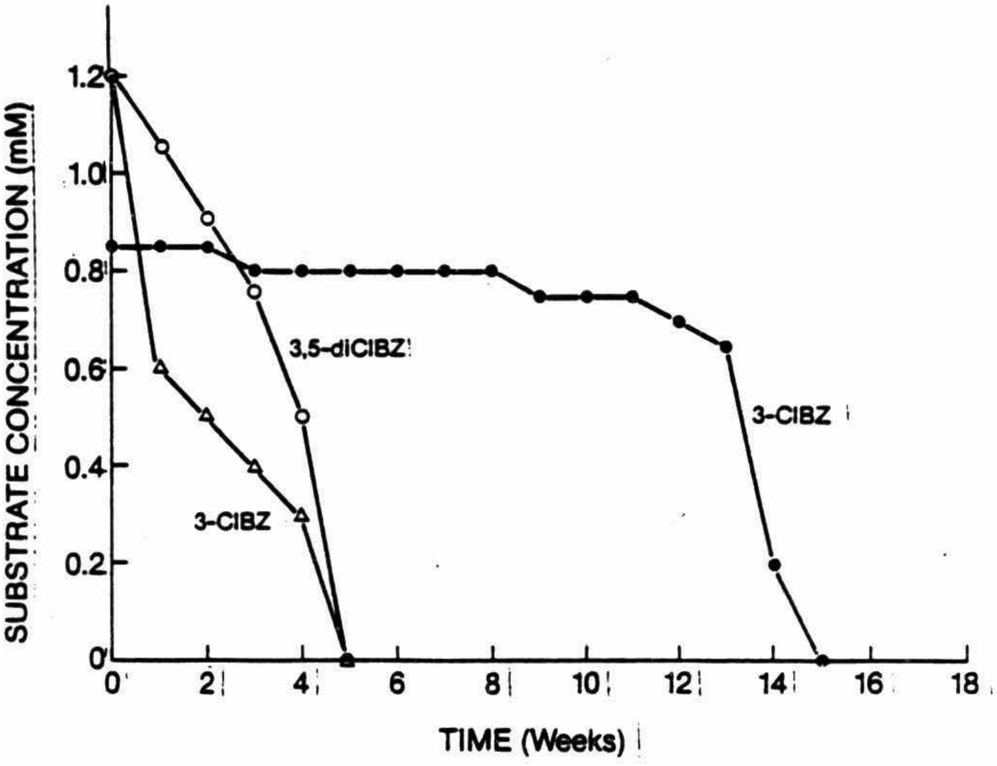

FIGURE 7A

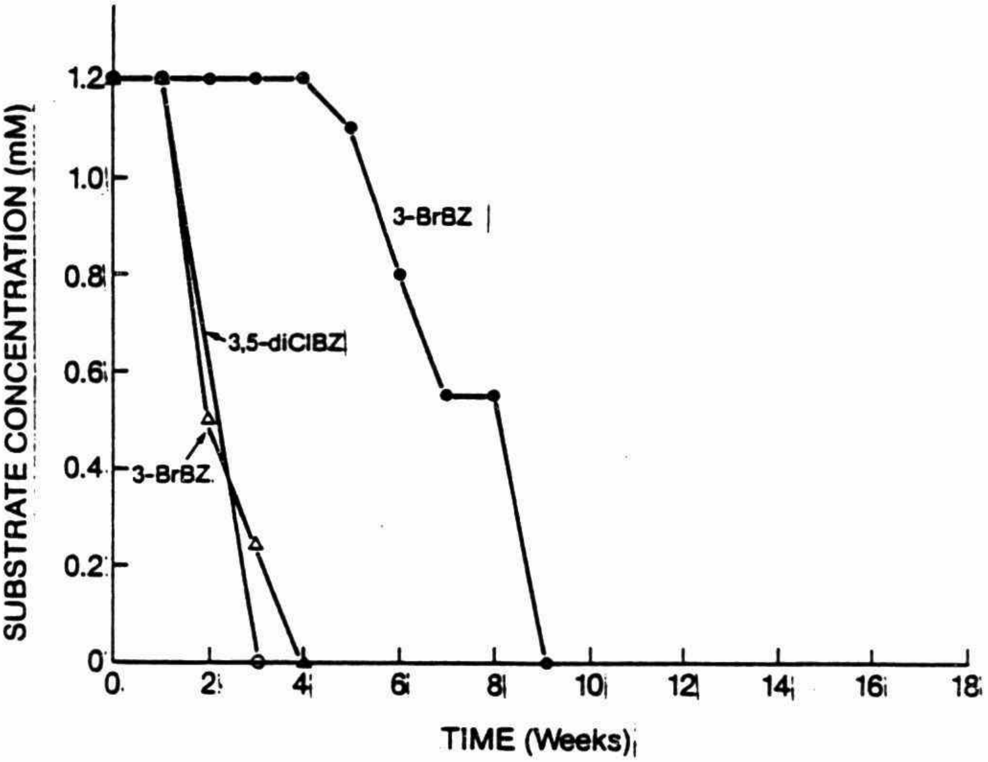

FIGURE 7B

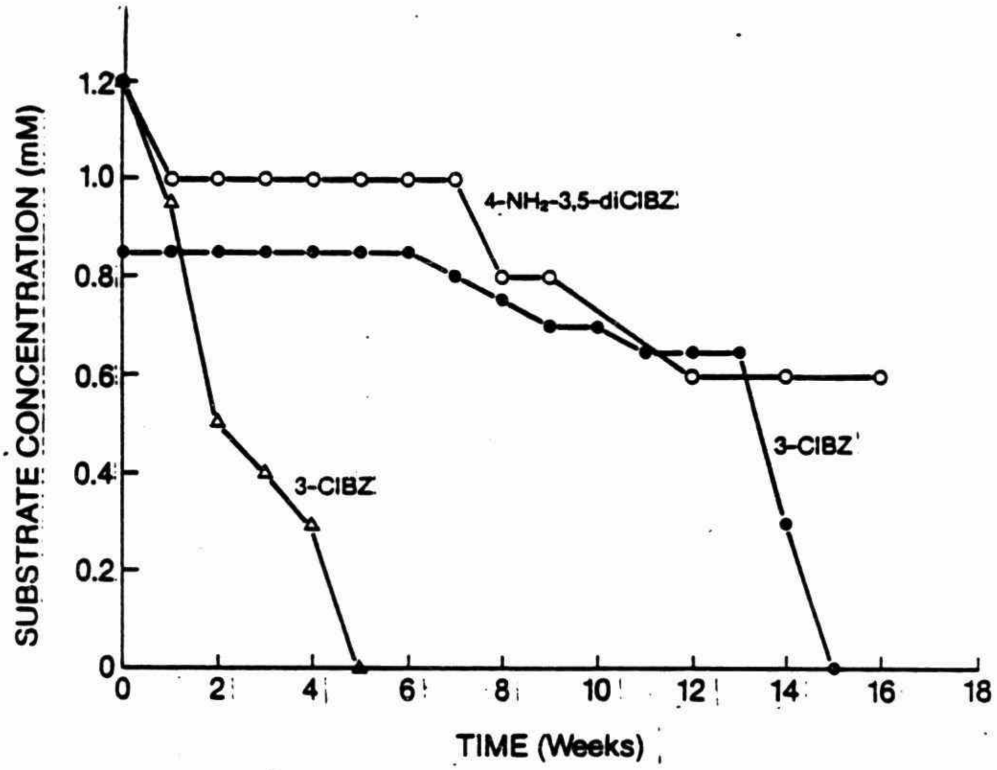

FIGURE 8A

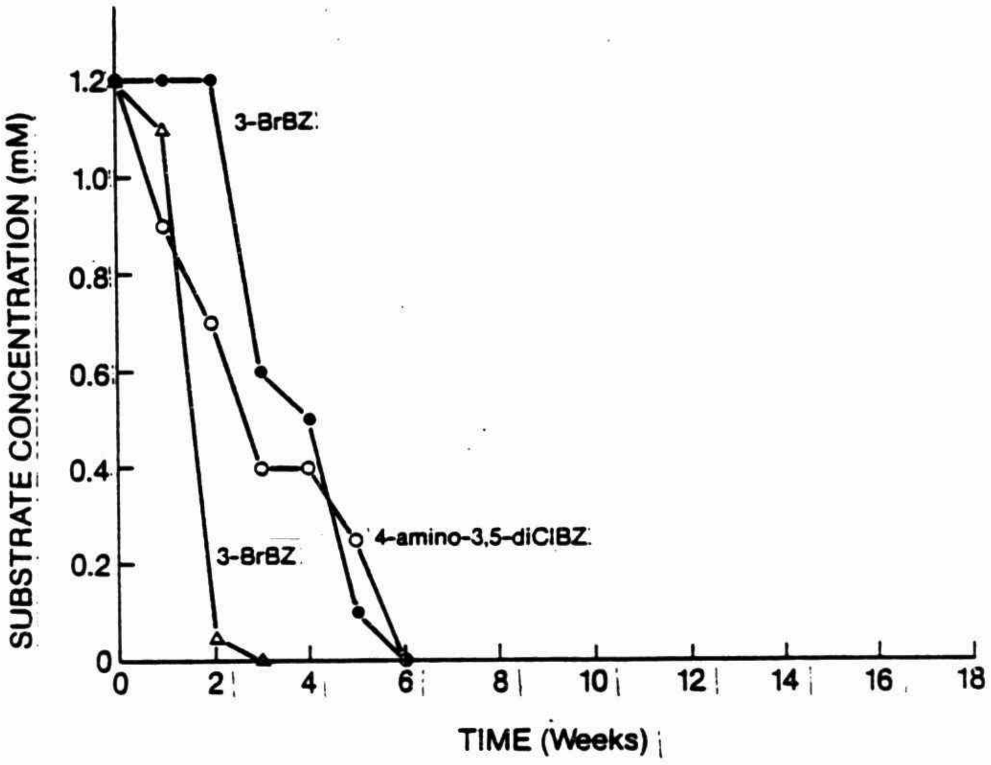

FIGURE 8B

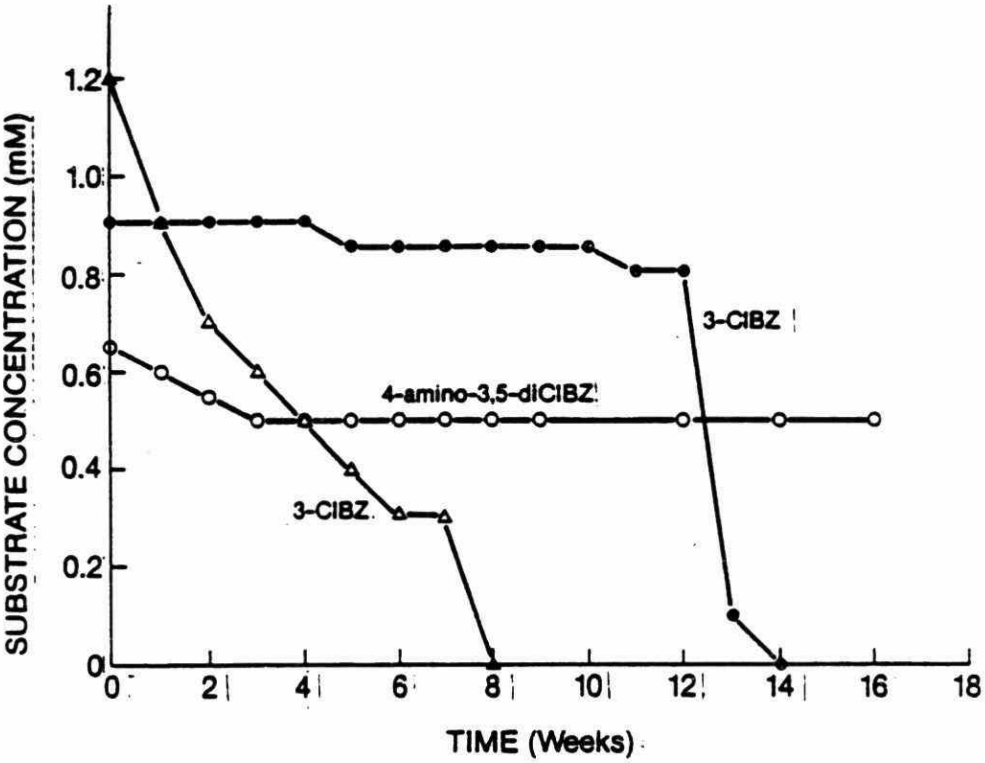

FIGURE 10A

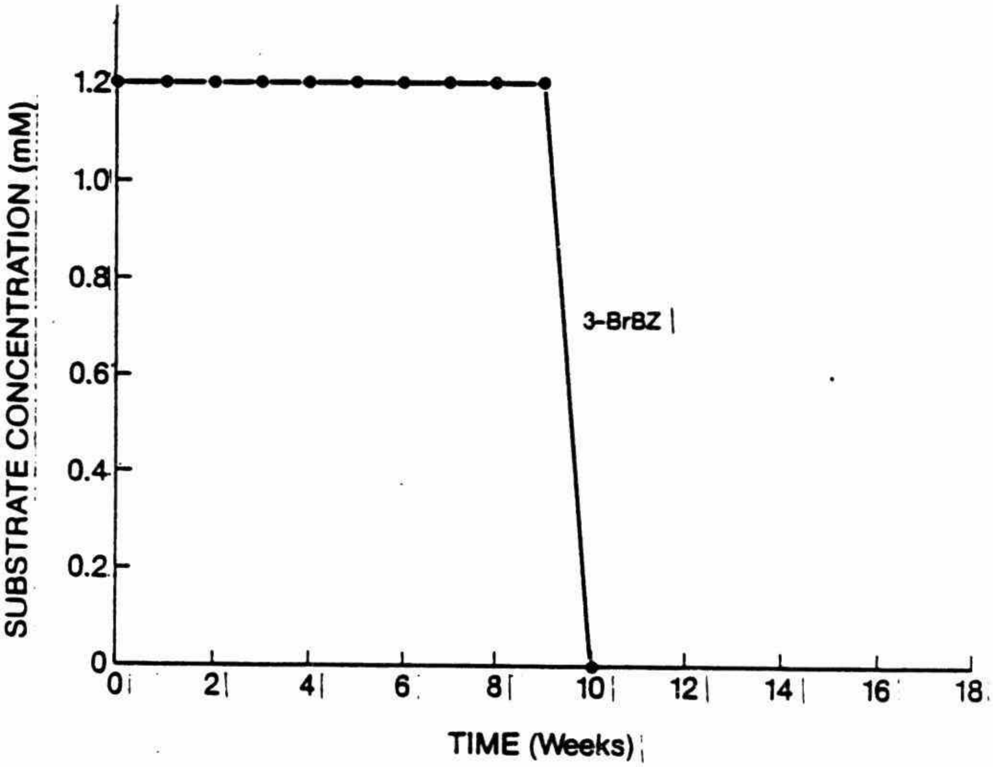

FIGURE 10B

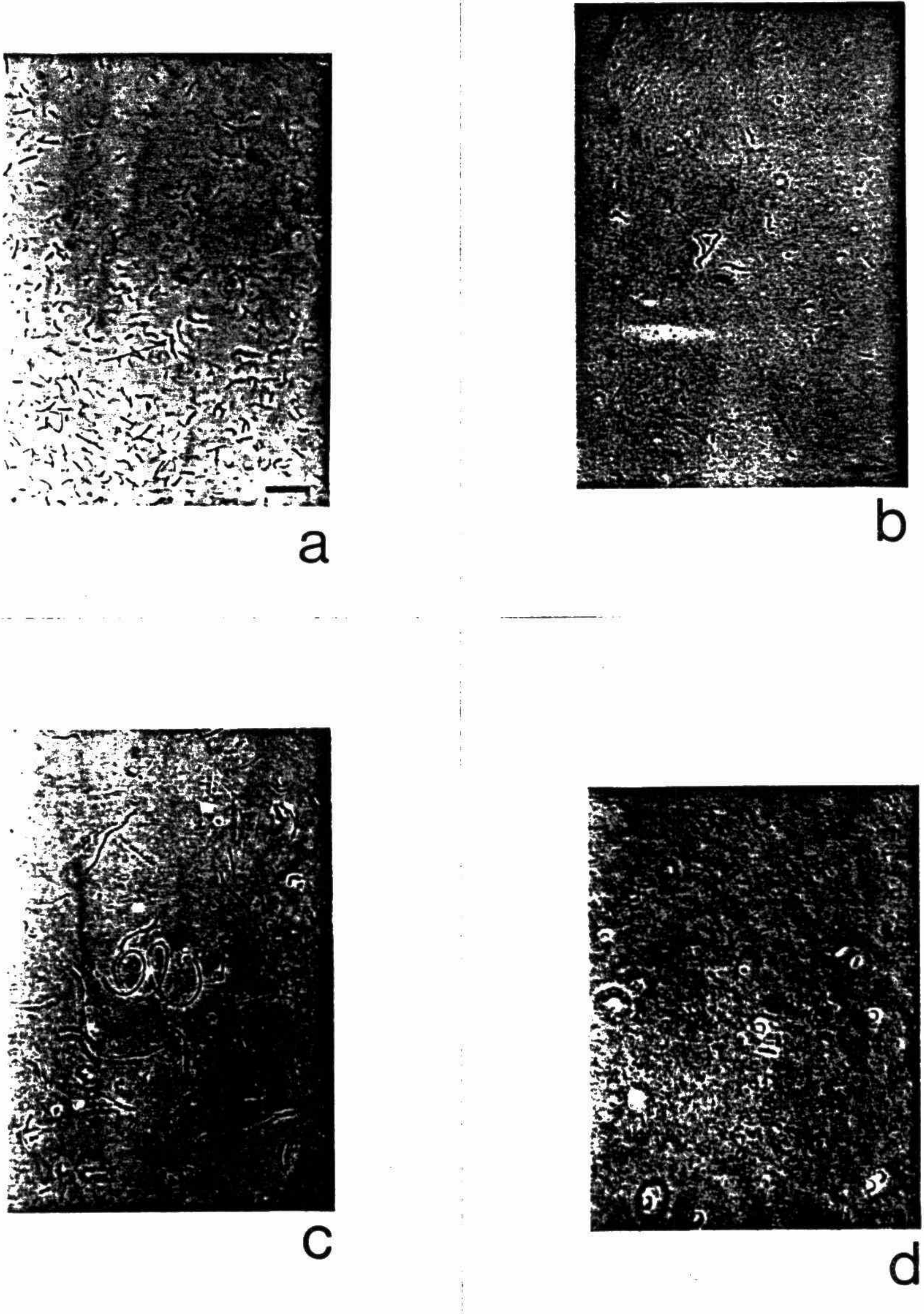

FIGURE 11